Supplementary biotin decreases tibial bone weight, density and strength in riboflavin deficient starter diets for turkey poults

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Sort title: RIBOFLAVIN AND BIOTIN IN TURKEY POULTS

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Abstract 1. Growth and skeletal responses to different dietary concentrations of riboflavin and biotin were compared in turkey poults from hatch to 21 d of age. The birds were fed on a turkey starter diet with different concentrations of supplementary riboflavin (0, 20 and 40 mg/kg) and biotin (0, 0.3, 0.6 mg/kg) in a factorial design.

2. Poults fed on diets with no supplementary riboflavin had poor gait scores, decreased times to sit and higher rates of culling compared with poults fed on the control diet (20 mg biotin and 0.3 mg riboflavin/kg diet). Histologically, riboflavin deficiency was associated with a peripheral neuropathy similar to that described previously in chicks and, unexpectedly, in growth plate abnormalities.

3. Tibiae of poults fed on the control diet were larger, more dense, stronger and stiffer than the diets with no supplementary riboflavin.

4. Increasing supplementary biotin in poults fed on diets with no supplementary riboflavin was associated with a decrease in tibia weight, density, strength and stiffness

5. The results demonstrated that riboflavin deficiency in fast growing turkey poults was associated with growth retardation, growth plate disturbance and peripheral nerve dysfunction leading to an inability to walk.

INTRODUCTION

Riboflavin and biotin are two water soluble vitamins required for energy metabolism and optimum growth. Deficiency of both vitamins has been associated with a variety of clinical signs including growth retardation, neurological, skeletal and skin abnormalities in chicks and turkey poults. Riboflavin is essential for healthy skeletal growth and locomotor function and biotin is

necessary for healthy skin and bone development (Ruiz and Harms, 1988, 1989)**.** However, little work has been reported on interactions between biotin and riboflavin in commercial avian species. Patrick *et al*., (1944) studied both of these vitamins in turkeys and found no evidence of an interaction between them but there appears to be no later publications that assess both vitamins simultaneously in turkey poults. The objective of the research was therefore to reassess the hypothesis that biotin and riboflavin do not interact to affect the growth and skeletal health of turkey poults from 0 to 3 weeks of age.

MATERIALS AND METHODS

Animals and husbandry

A total of 216 Hybrid Grade Maker male turkey poults (Hybrid Turkeys, Kitchener, Canada) were obtained from a commercial hatchery. The poults were housed in 36 pens in groups of 6 and brooded under ceramic heat lamps at conventional house temperatures of 25-27^{o}C. Each pen was 1.45 m wide x 2.45 m deep and was littered with wood shavings. The birds were presented with a photoperiod of 16 h light and 8 h dark after the first 24 h at an intensity of 20 lux at bird head height. Each pen contained a suspended drinker and a pan feeder and both water and feed were freely available throughout the experiment.

A standard turkey starter diet lacking riboflavin and biotin in the vitamin supplement was used as the basal diet (Table 1) to which was added the supplementary vitamins. Dietary vitamin supplementations respectively were 0, 20 and 40 mg/kg for riboflavin and 0, 0.3 and 0.6 mg/kg biotin in a 3 x 3 factorial arrangement of 9 diets.

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66 **Live bird observations**

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68 Body weight was recorded at hatch and at 7, 14 and 21 days of age. Weekly feed consumption
69 was measured at 7, 14 and 21 d from which daily feed intake was calculated as the total feed
70 consumed divided by the number of bird days.

71 Each poult was identified by a wing band at hatch and by a within-pen code applied by
72 a black felt tip pen on its back for ease of identification during behavioural observations. The
73 gait of each poult was scored on days 12, 14, 16 and 19 by the same observer. The observer
74 entered the pen and walked around the perimeter gently driving the poults before them. Walking
75 ability was scored on a 4 point scale: normal (0), mild gait disturbance (1), clear gait defect with
76 slow movement but minimal effect on the ability to feed (2), and obvious difficulty moving (3).
77 Score 3 was also given to poults that were culled at or before the time of the observation. The
78 gait score of each poult was recorded by an assistant directly into an Excel worksheet. At the
79 completion of gait scoring on days 14, 16 and 19 the same procedure was followed starting once
80 more at the first pen except that the observer left the pen, closed the door, and observed the birds
81 for up to 120 s. The time from when the observer exited the pen for each bird to sit was recorded
82 using a purpose-built Excel macro by the assistant (however, it was not possible to observe the
83 bird number). Observations for birds that had not sat during this time were recorded as unknown.

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85 **Morphology and histopathology**

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All poults from each pen fed on the unsupplemented riboflavin diets and the "control" pens receiving 20 mg riboflavin and 0.3 mg biotin/kg were weighed and culled for detailed investigation of skeletal morphology and histopathology on day 19. Two randomly selected birds from each of the other pens were sampled on day 20. Each bird was euthanized using an intravenous lethal dose of sodium pentobarbitone and weighed. The external appearance of each bird was assessed and the presence of feather curling and foot pad lesions were recorded. A sample of the skin from the right ventral abdomen and right foot pad was taken into neutral buffer formalin (NBF, phosphate buffered formalin, pH7.3-7.4, Genta Medical, Tockwith, UK) for histological analysis. The body cavity was opened and the internal organs were examined. A sample of right caudal lung, liver, gizzard, spleen, duodenum, terminal ileum, right sciatic nerve, muscle from the right thigh, right axillary nerve and 3 cm of lumbosaecral vertebrae and spinal cord were fixed in NBF. The right stifle joint, including distal femur and proximal tibia, was fixed in NBF. The left tibia was stored unfixed for morphometric analysis (see below). The remaining birds were weighed and killed by cervical dislocation on termination of the experiment at 21 days of age.

Tissues from a random selection of birds from each of the 9 diets (2 from each diet: total 18 birds) and 8 birds culled prior to day 19 from diets with no supplementary riboflavin (consisting of 4, 1 and 3 birds respectively fed on the diets with 0, 3 and 6 mg supplementary biotin/kg), were processed using routine methods to histological sections and stained with haematoxylin and eosin. Slides were examined blindly and scored by one researcher (P.B.). Measurements of the width of the proximal tibial growth plate, primary and secondary spongiosa were taken for all 26 birds under a x2 magnification using the Cell Imaging Software (Olympus). A random sample of slides from affected and normal poults were subsequently stained with

Holmes/Luxol fast blue method (Culling *et al.*, 1985) to visualise the axons and myelin sheaths of the peripheral nerves and spinal cord.

The morphology of the left tibiotarsus was assessed on 8 of 9 birds that were culled and all poults sampled on days 19 and 20 (2 birds/pen). The tibiae were weighed and radiographed on the lateral plane in a Faxitron Soft X-ray Cabinet (Faxitron Bioptics, IL, USA) alongside a 16 step aluminium calibration step-wedge. Films were scanned into a PC (Dell Quad Core Processor) with a Kodak X-ray Line Scanner (Kodak, Hemel Hempstead, UK) and the total bone density, length and width were determined using image analysis software (ImageJ; URL: http://rsb.info.nih.gov/ij/). Bone density was determined by comparing the density of the image with that of the aluminium wedge and was defined as the equivalent density of aluminium of a specified depth (mm). The long axis of the bone was established by drawing a straight line from the proximal to distal extremities of the tibiotarsus and used to measure the length of the bone. The width of the tibia was measured at the midpoint of the diaphysis. Breaking strength and stiffness measurements were made on an LR50 materials testing machine (JJ Lloyd, Fareham, England) at a compression speed of 30 mm/min.

Welfare considerations

The experiment was conducted under relevant Government licences after local ethical review. Protocols for culling poults on the basis of daily inspection of behaviour and especially of walking ability were implemented to protect the welfare of the birds. The criteria for culling on welfare grounds was initially set at score 3 but changed to score 2 after 16 d because of the rapid deterioration of the condition of the affected poults.

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135 **Statistical analyses**

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137 The experiment was a randomised block design with 4 replicates (blocks) of 9 pens. Treatments
138 were randomised to pens and pen means were analysed by conventional ANOVA of a model
139 with effects for block, biotin, riboflavin and their interaction except as described below. Body
140 weight at 21 days for poults sampled at 19 and 20 days was obtained by linear extrapolation. The
141 proportion of birds culled or dying between 7 and 21 days was analysed with binomial errors in
142 the GLMM procedure of Genstat (2009). Time to sit could not be assigned to individual poults
143 and the proportion of surviving poults sitting after 90 s in each pen was analysed with a model
144 including age as a split plot effect and assuming binomial errors.

145 Gait scores for the 3 diets containing no supplementary riboflavin were analysed with a
146 model including age within bird as a split plot effect and Poisson errors. Tibial bone
147 measurements on all of the birds on these 3 diets and the control diet containing commercial
148 recommendations for both vitamins (20 mg riboflavin/kg and 0.3 mg biotin/kg) were analysed by
149 REML methods using a model with fixed effects for diets (4 levels) and sampling (3 levels:
150 culled 16-17 d, sampled on day 19 or 20) and the random effects of block and pen within block.
151 The role of biotin was evaluated with the same model after omitting pens fed on the control diet.
152 The differences between the culled and sampled (surviving) poults in the same treatments were
153 assessed in a model with an additional factor (culled or not). Growth plate thicknesses were
154 analysed using REML of a model with bird as the random effect and biotin and riboflavin as
155 fixed effects.

156

157 RESULTS

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159 **Body weight and feed intake**

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161 The interaction of biotin and riboflavin on body weight was not significant at any age. Average
162 body weight of the poults was similar (mean 123 g) among different treatments at day 7. There
163 was a significant reduction in body weight at subsequent ages in the poults fed on diets with no
164 supplementary riboflavin but supplementary biotin had no effect. Mean body weights at day 14
165 for poults fed on diets containing 0, 20 or 40 mg/kg supplementary riboflavin were 249, 322 and
166 323 g (SED 7.7, $P<0.001$) and 302, 292 and 300 g (SED 7.7, not significant) for poults fed on
167 diets containing 0, 0.3 and 0.6 mg/kg biotin respectively. Marginal means for both vitamins at 21
168 d are presented in Table 2.

169 Daily feed intakes averaged 14 g/d in the first week. The effect of riboflavin on feed
170 intake was highly significant ($P<0.001$) during weeks 2 and 3. Average intakes for birds fed on a
171 diet with supplementary riboflavin in weeks 2 and 3 averaged 30 and 70 g/d whereas those fed
172 on the unsupplemented riboflavin diets with 0, 0.3 and 0.6 mg/kg biotin respectively consumed
173 less, at 22, 15 and 22 g/d (SED 4.8 g) in week 2 and 38, 39 and 48 g/d (SED 4.9 g) in week 3.

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175 **Culling and behaviour**

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177 A total of 6 birds died in the first week: these were assumed to be poults that had failed to initiate
178 proper feeding activity. Nine birds were culled on welfare grounds at 16 and 17 d. Culling was

strongly associated with riboflavin deficiency since 7 of the 9 culled birds were fed on the diet with no supplementary riboflavin or biotin. The two remaining poults were fed on diets supplemented with 0.3 mg biotin and either 20 or 40 mg/kg riboflavin. Culling and mortality was analysed as the proportion of birds present at day 7 that were subsequently culled or that died. The overall effect of supplementary riboflavin on culling was significant ($P<0.001$). The backtransformed mean (± SE) culling rates for poults fed on diets containing no supplementary riboflavin and 0, 0.3 and 0.6 mg/kg biotin respectively were 20.9±8.26, 13.5±7.23 and 8.6±5.81 % (not significant) compared with 2/141 (1.4%) in the other treatments. The remaining poults fed on the unsupplemented riboflavin diets were culled at 19 d.

Gait scores 2 and 3 were only observed in poults fed on diets with no supplementary riboflavin; on diets with supplementary riboflavin, a score 1 was observed in 1 individual at 12 d and another at 24 d. These 2 records were ignored and the data for individual gait score of birds fed on the riboflavin unsupplemented diets only were analysed. The mean gait score in birds fed on these diets at 12, 14, 16 and 19 d was 0.37, 1.05, 1.41 and 2.02 (SED 0.072, $P<0.001$) showing a deterioration in gait over time. For poults fed on diets with 0, 0.3 and 0.6 mg/kg supplementary biotin respectively mean gait scores were 1.26, 1.20 and 1.18 (SED 0.106, not significant).

The proportion of poults sitting down before 90 s was similar across ages: means (backtransformed, %) at 14, 16 and 19 d respectively were 0.39 (60), 0.45 (61) and 0.34 (58), (SED 0.292, not significant). The effect of the interactions with age and the marginal effect of biotin were not significant whereas the different concentrations of riboflavin had a large effect on time to sit (Table 2).

202 **Tibial bone morphology**

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204 Tibial bone weight, morphology and strength, were analysed for 72 randomly selected poults
205 (mean of 2 per pen), including those that were culled at 19 and 20 d. The vitamin interaction was
206 not significant for any trait except stiffness ($P<0.05$). Marginal means for tibial bone weight,
207 length, width, maximum load and density are presented in Table 2 and the means for stiffness are
208 plotted in Figure 1. The marginal effect of riboflavin was highly significant ($P<0.001$) and the
209 interaction was associated with the low stiffness of tibiae of poults fed on diets with no riboflavin
210 and 20 and 40 mg/kg biotin compared with diets with no supplementary biotin: marginal means
211 (SED 0.057) for 0, 20, and 40 mg/kg supplementary riboflavin were 11.19, 11.65 and 11.70
212 ($P<0.001$) ln N/m and those for 0, 0.3 and 0.6 mg/kg biotin were 11.53, 11.50 and 11.51 ln N/m
213 (not significant).

214 The analyses of these data were repeated with body weight as a covariate. The interaction
215 was not significant and there was little difference in the results for the biotin diets (data not
216 shown). Differences between the marginal means for supplementary riboflavin decreased
217 whereas the SED was increased by 30-80%. Nevertheless tibial bone length adjusted for body
218 weight was lower in the unsupplemented diet. Marginal means for 0, 20 and 40 mg
219 supplementary riboflavin/kg respectively were 72.7, 82.3 and 79.5 (SED 4.29, $P<0.05$) for
220 length; 7.44, 7.13 and 6.91 (SED 0.439) for tibial bone weight; 5.21, 5.51 and 5.44 (SED 0.321)
221 for width; 1.19, 1.17 and 1.17 (SED 0.028) for density; and 117.8, 123.2 and 123.6 (SED 8.79)
222 for maximum load.

223 There were data from an additional 58 birds from the control diet and the 3 diets with no
224 riboflavin supplement. The combined data set from these 4 diets contained 90 records (out of 96

at hatch) for analysis and 68 for the 3 diets that contained no supplementary riboflavin. The results of three statistical analyses of these data are summarised in Table 3. The tibiae of the birds fed on the control diet were significantly larger ($P<0.05$) and more dense, stronger and stiffer ($P<0.01$) than the diets with no supplementary riboflavin or biotin (Table 3 (a)). Tibial weight, density and strength were higher ($P<0.05$) in the poults fed on the diet with no supplementary riboflavin or biotin compared with those fed on diets with 0.3 and 0.6 mg biotin/kg which were similar (Table 3 (b)). The tibia of the 9 culled poults were significantly ($P<0.001$) smaller, less dense, weaker and with lower stiffness than those of survivors (Table 3 (c)).

Histology

The most striking changes in histological sections were observed in the peripheral nerves and growth plates of the birds fed on the diets containing no supplementary riboflavin. The changes in the peripheral nerves were segmental and characterised by an increase in the number and size of Schwann cells in the sciatic and axillary nerves accompanied in some samples by a mild to moderate increase in the number of mitotic figures. The myelin sheaths surrounding the axons appeared swollen and vacuolated (Figures 2 and 3) and there were mild to moderate perivascular lymphocyte and plasma cell infiltrations. The Holmes stain revealed a moderate amount of axonal fragmentation and degeneration.

The changes in the proximal tibial growth plate consisted of mild to moderate disorganisation of the chondrocyte columns in the growth plate (Figure 4) and shortened, thickened primary and secondary spongiosa. Occasionally horizontal clefts were present within

248 the growth plate. The interaction of biotin and riboflavin for the depths of growth plate, primary
249 or secondary spongiosa was not significant and the data were reanalysed omitting the interaction.
250 Marginal means are presented in Table 4. The effect of supplementary riboflavin was significant
251 (P<0.01) for all 3 regions whereas the effect of biotin was not. The unsupplemented riboflavin
252 diet was associated with a reduction in the depth of each measurement whereas the primary and
253 secondary spongiosa layers and the total depth were deeper in poults fed on 20 and 40 mg/kg
254 biotin.

255 Grossly, 2 of 19 birds fed on the unsupplemented diet had footpad lesions (Figure 2a).
256 Histologically the footpads of 2/5, 3/3 and 3/4 birds on diets containing 0, 0.3 and 0.6 mg/kg
257 biotin with no supplementary riboflavin showed hyperkeratosis and dyskeratosis (thickened and
258 abnormal keratinisation). This was accompanied in most affected birds by a perivascular
259 mononuclear and occasionally heterophilic dermatitis (Figure 5). In some birds the lesion had
260 progressed to ulceration of the epidermis.

261 In 7 birds there was evidence of mild to moderate, chronic, focal to multifocal,
262 lymphocytic and histiocytic pancreatitis noted predominantly in the birds fed on diets without
263 riboflavin supplementation (6/7). No significant findings were detected in the samples of lung,
264 duodenum, ileum, spinal cord or breast skin. A range of mild pathologies were identified in the
265 liver but not associated with any particular diet.

266 Slight curling of the wing feathers was noted grossly in 6 poults fed on the diet
267 containing no supplementary riboflavin or biotin (3 culls and 3 sampled at termination).

268

269 DISCUSSION

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Riboflavin (or vitamin B2) is a water soluble vitamin which acts as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucelotide (FAD) which are both important as co-factors in a wide variety of different enzyme systems, especially those involved in energy metabolism. Biotin (or vitamin B7) is also a water soluble vitamin and is a coenzyme in a number of carboxylases that act as key enzymes in the metabolism of glucose, fatty acids and leucine. Both vitamins are required for optimum growth of poultry. This study was designed to investigate a previous finding (Patrick *et al.*, 1944) that there was no association between biotin and riboflavin in grower diets for turkey poults. Poults were fed a control diet, and diets with a reduced or increased supplementation of biotin and riboflavin. The most obvious clinical signs were seen in the poults fed on diets without supplementary riboflavin. At 12 d some of the poults exhibited a crouched stance with wings lower that the hocks. They showed instability when standing, were slower, and tended to sit more quickly than unaffected poults. These signs progressed to a wide stance and definite varus gait at 14 d with both limbs and wings extended laterally. Severely affected poults were culled at 16 d and 17 d followed by premature termination on welfare grounds at 19 d of all the poults fed on the diets with no supplementary riboflavin. The welfare of poults with a gait score of 2 was observed to decline rapidly so that they were unable to walk or feed and drink within 12-24 h.

The average body weight of the birds that were culled on humane grounds at 16 and 17 d was similar to that of the remaining birds fed on the same diets at 7 (126 vs 118, SED 6.1 g) and 14 days of age (256 vs. 248 g, SED 15.0 g). Furthermore the mean (±SE) body weight gained in the culled birds from 14 to 16 or 17 d was 46±5.4 g which was also similar to the weight gained by the surviving birds (42±2.0 g), indicating that there was no association between body weight or weight gain and culling.

The diets were presented as a coarse mash because of the difficulty of pelleting small quantities and both feed intake and body weight were lower than breed standards for male poults (http/:www.hybridturkeys.com, accessed 12th December 2011). However, poult weight at hatch was also relatively low at 53 g compared with an expected weight of 60 g and breed targets for body weight at 7, 14 and 21 days of 150, 370 and 710 g compared with 124, 324 and 642 g for poults fed on the control diet. It is therefore likely that the adverse effects of the unsupplemented riboflavin diets would be observed at a younger age or to a more extreme degree in commercial poults achieving target growth rates. Whereas reduced growth associated with riboflavin and biotin deficiency was identified in previous research (e.g. Bain *et al.*, 1988; Ruiz and Harms, 1989), classical signs of riboflavin and biotin deficiency such as perosis, curled toe paralysis or encrustation at the corner of the beak and around the eyes were not evident. In general, feathering was not grossly affected in poults fed on the riboflavin deficient diets, possibly because the experiment finished before these symptoms had developed. Foot pad dermatitis was observed in several poults fed on the riboflavin deficient diets with supplementary biotin suggesting that supplementary riboflavin may be as important as biotin for foot pad health: no evidence for an effect of supplementary biotin on the prevalence of foot pad dermatitis above a basal concentration of 200 µg/kg was reported in an earlier experiment (Mayne *et al.*, 2007).

It is likely that stores of maternal riboflavin in the yolk sac were metabolised in the first 10 days after hatching and that deficiency symptoms became evident from 12 d of age as altered gait associated with degenerative changes in the myelin sheath of the peripheral nerves after maternally derived sources of riboflavin had been exhausted. The peripheral demyelinating neuropathy identified in the riboflavin deficient poults in this study was comparable with the pathology reported by other workers in young broiler chickens (Cai *et al.*, 2007, 2009; Cai *et al.*,

2006a; Cai *et al.*, 2006b; Johnson and Storts, 1988). Similar to the disease in broiler chickens, riboflavin deficiency in turkey poults affects the peripheral nerves and leaves the spinal cord unaffected. The lesions clearly explain the paralysis and lowered wings in affected poults and suggest that the wings are not used for support during attempts at walking but are a result of the primary lesion in the axillary nerve. No lesions were identified in the smaller subcutaneous nerves in the poults fed the riboflavin deficient diet, supporting the theory of Cai *et al.* (2009) that riboflavin deficiency in young birds produces selective injury to large peripheral nerve trunks while sparing the smaller, more distal nerves. No trend was identified in the pathology present in the peripheral nerves in the poults fed no riboflavin and increasing levels of biotin; however the segmental nature of the lesions hampered quantitative assessments. The results of the pathology analysis are consistent with the behavioural evidence that both mean gait score and time to sit were adversely affected in poults fed on diets with no supplementary riboflavin whereas biotin supplementation had no effect.

In addition to the neuropathology, lesions were also identified in the growth plate of riboflavin deficient poults. Pathology in the skeleton has not been reported in previous studies of young broiler chickens fed a riboflavin deficient diet, and a link between riboflavin deficiency and bone growth is not well documented in any species, suggesting the changes seen in the bones and growth plates of the poults in this study could be peculiar to turkeys. The pathological changes identified were not consistent with a deficiency of either calcium or phosphorus. Further analysis of riboflavin levels and skeletal disease in turkey poults is indicated.

Bain *et al.* (1988) fed chicks biotin deficient diets and showed that they had higher bone densities and percentage bone ash and shorter lengths of tibiae compared with chicks fed on a diet with an adequate concentration of biotin and suggested that the decrease in length was

proportional to the retardation in body weight. These results contrast with the lack of any difference in the turkey poults fed on diets with supplementary riboflavin and no supplementary biotin (data not shown).

Whereas differences in luxol fast blue staining of the myelin (Figure 3) were suggestive of an effect of biotin on the sciatic nerve, there were large differences between samples and a definitive conclusion regarding the role of biotin in preserving nerve function could not be made.

In conclusion, there was no evidence of an interaction between riboflavin and biotin at concentrations up to twice that recommended provided that riboflavin was added to the diet. A deficiency of riboflavin produced a rapid response in the poults after 10 d of age that was characterised by inappetance, poor growth, reduced size and organisation of the tibial growth plate, ataxia and increasing paralysis of the limbs and wings associated with defective myelination of the sciatic and axillary nerves. Increasing concentrations of dietary biotin in riboflavin deficient diets were associated with a decrease in tibia weight, density and strength. It is possible that an excess of biotin interferes with riboflavin absorption and increases the severity of riboflavin deficiency and this also should be investigated. The results are consistent with the proposal of Ruiz and Harms (1988) that symptoms of riboflavin deficiency are more severe in fast growing modern strains than traditional lines, an observation that may explain the conclusion that there was no interaction between riboflavin and biotin in turkey poults in an experiment performed in the 1940s (Patrick *et al.*, 1944).

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Legends for figures

Figure 1. *Mean stiffness (SE bar) of tibia from turkey poults at 21 d of age fed on diets with different concentrations of riboflavin and biotin.*

Figure 2. *Sciatic nerve of turkey poults at 21 d of age. A. Poult (#1613) fed on a diet with no supplementary riboflavin and biotin showing an increase in the number and size of Schwann cells. The myelin sheaths surrounding the axons are swollen and vacuolated. B. Normal sciatic nerve from a poult (#1491) fed on the control diet with adequate riboflavin (20 mg/kg) and biotin (0.3 mg/kg). H&E stained sections, x10 magnification.*

Figure 3. *Sciatic nerve of turkey poults at 21 d of age. A. Poult (#1511) fed on a diet with no supplementary riboflavin and 0.6mg biotin/kg showing less myelin (characterised by paler blue staining of the section). Nerve fibres can be seen staining grey (arrow). B: Normal sciatic nerve from a poult (#1491) fed on the control diet with adequate riboflavin (20 mg/kg) and biotin (0.3 mg/kg). Holmes Luxol Fast Blue stain, x20 magnification.*

Figure 4. *Proximal tibial growth plate of turkey poults at 21 d of age. A. Narrow growth plate and disorganised chondrocytes in a poult (#1602) fed on a diet with no supplementary riboflavin or biotin. B. Proximal growth plate of a poult (#1491) fed on the control diet with adequate*

riboflavin (20 mg/kg) and biotin (0.3 mg/kg) with a deeper growth plate and vertically oriented columns of chondrocytes. H&E stained, x4 magnification.

Figure 5. *Junction of dermis and epidermis in the footpad of a turkey poult at 21 d of age fed on a diet with no supplementary riboflavin or biotin (#1454). The section shows an infiltration of red, granular heterophils (H) within the dermis, accompanied by macrophages and a smaller number of lymphocytes. There are foci of pyknotic cells in the epidermis (P). H&E stained section, x20 magnification.*

424 **Table 1**. Basal diet vitamin and mineral composition.

425

Ingredient	g/kg
Wheat	505.3
Maize gluten (600 g CP/kg)	50.0
Soya bean meal (480 g CP/kg)	200.0
Full fat soya (380 g CP/kg)	100.0
White fish meal (650 g CP/kg)	100.0
L-lysine	3.0
DL-methionine	2.5
Soya oil	15.0
Limestone (Trucal 52)	5.0
Monocalcium phosphate	12.5
Sodium chloride	1.5
Sodium bicarbonate	0.5
Vitamin-mineral premix[1]	4.0
Elancoban[2]	0.5
Ronozyme WX[3]	0.2
Calculated composition	
Energy, MJ ME	12.8
Crude protein	290
Calcium	12.7
Phosphorus	10.1

[1] Supplying retinyl acetate 4.09 mg/kg, cholecalciferol 125 mcg/kg, α-tocopherol 100 mg/kg, menadione 4 mg/kg, thiamine 5 mg/kg, pyridoxine 7 mg/kg, cobalamin 0.04 mg/kg. niacin 150 mg/kg, pantothenic acid 25 mg/kg, folic acid 4 mg/kg, ascorbic acid 200 mg/kg, choline chloride 1200 mg/kg; I 2 mg/kg, Se 0.2 mg/kg, Cu 20 mg/kg, Fe 50 mg/kg, Mn 120 mg/kg, Zn 100 mg/kg, Co 0.5 mg/kg, Mo 0.5 mg/kg.

[2] Monensin sodium coccidiostat, Elanco Animal Health, Basingstoke, UK.

[3] Xylanase enzyme, DSM Nutritional Products Ltd., Basel, Switzerland.

435 **Table 2.** Marginal means for body weight, tibial bone length and density and time to sit in turkey
436 poults aged 3 weeks fed on a starter diet containing different concentrations of riboflavin and
437 biotin. (Means are for 12 pens of 6 poults).

438

Vitamin	Body	Left tibia					Sitting
	weight, g	Weight	Length	Width	Density	Maximum	<90 s,
		g	mm	mm	mm Al	load N	prop.[1]
Biotin, mg/kg [2]							
0	565	7.27	76	5.26	1.18	123	0.47 (61)
0.3	564	6.92	80	5.49	1.18	120	0.12 (53)
0.6	569	7.40	80	5.47	1.18	124	0.59 (64)
Riboflavin, mg/kg							
0	410	5.92	68	5.03	1.44	94	1.86 (87)
20	633	7.85	85	5.63	1.20	134	-0.13 (47)
40	635	7.82	82	5.55	1.20	139	-0.55 (37)
Significance[3]	***	***	***	**	***	***	***
SED	16.2	0.346	2.8	0.204	0.015	6.7	0.390

439

440 [1] Proportion of birds sitting after 90 s (backtransformed, %).

441 [2] Differences between biotin concentrations were not significant.

442 [3] Differences between concentrations of riboflavin: *** $P<0.001$; ** $P<0.01$.

443

444

445 **Table 3.** Marginal means of tibial bone traits in the "control" diet and 3 diets containing no
446 supplementary riboflavin and different concentrations of biotin. Statistical tests are presented for
447 (a) comparisons among these diets, (b) differences among these diets alone and (c) between
448 culled and surviving poults. (Means are for 4 pens of 6 poults)

Diet	Weight	Length	Width	Density	Max Load	Stiffness
	g	mm	mm	mm Al	N	ln N/m
Control[1]	6.8	80.2	5.13	1.18	126	11.5
0 mg biotin/kg	5.8	66.2	4.90	1.13	99	11.2
0.3 mg biotin/kg	4.6	64.3	4.63	1.06	76	11.0
0.6 mg biotin/kg	4.7	65.2	4.78	1.09	81	11.5
(a) All 4 diets						
SED	0.37	1.72	0.118	0.017	6.6	0.08
Significance	*	***	*	**	***	**
(b) Between diets with no riboflavin and increasing biotin						
SED	0.44	2.01	0.117	0.018	7.5	0.10
Significance	**	NS	NS	*	*	NS
(c) Culled vs. survivors in diets with no riboflavin and increasing biotin						
Mean survivors	5.7	68.3	5.11	1.13	98	11.2
Mean culled	3.7	58.4	4.07	1.01	62	10.7
SED	0.53	2.77	0.236	0.030	9.0	0.100
Significance	***	***	***	***	***	***

449 NS not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

450 [1] Diet containing 20 mg riboflavin and 0.3 mg biotin/kg.

451 **Table 4.** Marginal means for the depth of the growth plate, and the length of the primary and
452 secondary spongiosa in turkey poults aged 3 weeks fed a starter diet containing different
453 concentrations of riboflavin and biotin.
454

Vitamin	Number	Growth plate, mm	Spongiosa, mm	455
			Primary	Secondary
Biotin, mg/kg				
0	10	1.45	5.05	2.89
0.3	7	1.33	4.46	2.68
0.6	9	1.40	4.46	2.58
Significance[1]		NS	NS	NS
Riboflavin, mg/kg				
0	13	1.01	2.22	1.42
20	7	1.61	5.49	2.96
40	6	1.57	6.27	3.76
Significance		***	***	**
Average SED		0.103	0.442	0.634

456

457 *** $P<0.001$; ** $P<0.01$, NS not significant.